REMARKS/ARGUMENTS

Claims 1-7 and 15-21 are pending. Claims 1, 7, 15 and 21 have been amended. No new matter has been introduced. Reexamination and reconsideration of the present application is respectfully requested.

Claim Rejections

Claims 1-7 and 15-21 are pending. Claims 8-14 and 22-28 were previously withdrawn pursuant to a Restriction Requirement and the Applicant now cancels those claims, reserving the right to later file one or more divisional applications directed to the subject matter of the non-elected/cancelled claims. Claims 1, 7, 15 and 21 have been amended.

The Examiner contacted the Applicant on August 8, 2006 and August 9, 2006, to discuss possible amendments that could place the claims in condition for allowance. In the telephone call of August 8, 2006, the Examiner suggested withdrawing the canceled claims and amending the language of claims 1 and 15 to recite "providing a detectably labeled probe comprising a nucleotide sequence of SEQ ID NO:5...." In the telephone call of August 9, 2006, the Examiner suggested deleting SEQ ID NO:3 and SEQ ID NO:4 from the claims as SEQ ID NO:5 only hybridizes to SEQ ID NO:1 and SEQ ID NO:2.

The Applicant has made the suggested changes and respectfully submits that the above objections are overcome.

35 U.S.C. §112, Second Paragraph

The Examiner rejected claims 1-7 and 15-21 as "being unclear" under 35 U.S.C. 112, second paragraph. First, the Examiner noted that it is unclear how "represented by" limits SEQ ID NO:5. The Applicant has made the amendments suggested by the Examiner.

The Examiner also stated that it is unclear how to use a protein microarray with the DNA sequences claimed in claims 1 and 15. Applicant has amended the claims to remove "protein microarray."

Thus, the Applicant respectfully submits that the above rejections have been overcome.

35 U.S.C. §112, Enablement

The Examiner rejects claims 1-7 and 15-21 under 35 U.S.C. 112, first paragraph, as not

providing enablement for the claimed methods. The Examiner notes that, while the specification is enabling for a method for detecting and determining the quantity of bacteria that oxidizes ammonia to nitrite comprising SEQ ID NO:1 and 2 by detecting the labeled probe of SEQ ID NO:5, it does "not reasonably provide enablement for a method for detecting and determining the quantity of bacteria that oxidizes ammonia to nitrite comprising at least 96% identity over the full length of SEQ ID NO:1 and 2 by detecting the labeled probe of SEQ ID NO:5." The Applicant respectfully submits that the rejections are overcome in light of the amendments and arguments presented.

Furthermore, there are many existing patents that are directed to specific sequences and their variants. The issuance of these patents demonstrates that specifications setting forth particular sequences and describing particular variants enable one skilled in the art to make and use the sequence and its described variants. Thus, it is common in the art that patents disclosing nucleotide sequences also disclose and claim variants of those sequences (see, e.g. U.S. Patent Nos. U.S. Patent Nos. 6,825,002 and 6,573,066). Methods for comparing the similarity of two or more nucleotide sequences are well known in the art. Similar sequences are often identified using computer programs such as BESTFIT and BLAST (see, specification page 22-23). Further, hybridization may be used to detect the similarity between variant sequences and a reference sequence. Thus, one skilled in the art would be able to easily synthesize and identify nucleotide sequences that are variants of a reference sequence by using known techniques. Therefore, a specification that describes a nucleotide sequence and its variants allows one skilled in the art to make and use that sequence and its variants.

In addition, one of skill in the art understands that the 16S rDNA is a highly conserved region of gene, and has been used by those skilled in the art to discern and describe phylogenetic relationships, rather than functional comparisons, which impart information regarding which regions of the 16S rDNA are variable at the species level (Woese *et al.*, Proc. Nat'l. Academy Sci. 74(11): 5088-5090 (1977)). As such, it is known to one of skill in the art which regions of the 16S rDNA gene are universally conserved between species. The specification teaches how this concept is used to construct probes that target a specific homology to the 16S rDNA which determines the phylogenetic relationship of the bacteria with that sequence. Pages 26-28.

The use of 16S rDNA to determine phylogenetic relationships of species is well-known in the art (Teske *et al.*, J. Bact. 176(21): 6623-6630 (1994)). In Teske *et al.*, the 16S rDNA

sequences of ammonia- and nitrite-oxidizing bacteria are compared in order to show phylogenetic relationships. By sequencing that gene of known AOB and NOB, it was shown that almost every known AOB fell into the beta-subdivision of the *Proteobacteria* but that these bacteria could be distinguished from other beta-subdivision non-AOB based on the 16S rDNA sequences. This is important to show that beta-subdivision AOB form a group within the beta-*Proteobacteria* that includes no other non-AOB, while different species of AOB can be distinguished based on their 16S rDNA sequence.

The data presented in the present specification shows that SEQ ID NO:1 and 2 and variants thereof are discovered to fit into the larger beta-subdivision AOB group and thus one of skill in the art would recognize an organism identified by SEQ ID NO:1 or 2 or variants thereof as an ammonia-oxidizing bacteria. The Examples show that the isolated and purified organism does indeed oxidize ammonia and thus confirms this designation. The comparison of the known 16S rDNA sequences in Teske *et al.*, by aligning the sequences, demonstrate that only very specific regions of the bases vary. As mentioned above, by using the framework developed by Teske *et al.* and known tools such as BLAST, it was discovered that SEQ ID NO:1 and NO:2 are similar in the regions shared among known AOB but varies in those specific regions that distinguish AOB species (4% or less). This phylogenetic comparison is shown in Figure 1 of the specification. Accordingly, the Applicant respectfully submits that claims 15-21 are properly enabled.

However, to further examination, the Applicant has amended the claims to include the hybridization conditions. Support for the amendments can be found on pages 28-29 of the specification, where probe and hybridization conditions are disclosed. What is disclosed in the specification is well within the capabilities of one of skill in the art to make and use the invention as claimed. The specification discloses how the probes are developed and the condition parameters under which hybridization occurs. Page 27, Example 6. For example, the specification states "[i]n situ hybridization of the fixed, immobilized cells was carried out in a hybridization solution consisting of 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% sodium dodecyl sulphate (SDS), 25 ng of oligonucleotide probe, and varying amounts of formamide...To achieve the same stringency during the washing step, as in the hybridization step, the wash solution contained 20mM Tris/HCl (pH 7.4), 0.01% SDS, 5 mM EDTA, and NaCl. The concentration of NaCl varied according to the percent formamide used in the

solution. For 20% formamide the NaCl concentration was 215 mM, for 30% it was 120 mM, and for 40% the NaCl concentration was 46 mM...The optimum stringency was determined to be 30% formamide for the S-G-Nsspa-0149-a-A-18 probe. For the S-G-Nsspa-0149-a-A-19 probe the optimum stringency was determined to be 20% formamide. The optimum stringency was determined to be 20% formamide for the probe represented by SEQ ID NO:21, and 20% formamide for the probe represented by SEQ ID NO:24." Page 29, lines 2-23. Thus, one skilled in the art would be able to easily synthesize and identify nucleotide sequences that are variants of the reference sequences by using the specified hybridization conditions together with well-known techniques to practice the invention. These methods and techniques are routinely practiced in the art (*e.g.*, alignment search tool (BLAST) (S.F. Altschul et al. 1990. Basic local alignment tool. J. Mol. Biol. 215:403-410) and CHECK_PROBE (B.L. Maidak et al. 1994. The ribosomal database project. Nucleic Acids Res. 22:3485-3487.)). Thus, given what is taught about SEQ ID NO:1 and NO:2 in the specification, the specified hybridization conditions that are required, and the examples demonstrating how ammonia-oxidation level is analyzed, one skilled in the art would be able to readily synthesize and identify the variants of the reference sequence which share the claimed homology and also exhibit ammonia-oxidation.

In light of the above considerations, Applicants respectfully submit that the present claims meet the enablement requirement of patentability. It is therefore respectfully requested that the Examiner's rejection of claims 1-7 and 15-21 based upon the enablement requirement of patentability be withdrawn.

35 U.S.C. §112, Written Description

The Examiner rejects claims 15-21 under 35 U.S.C. 112, first paragraph, as not complying with the written description requirement. The Applicant respectfully submits that the rejections are overcome in light of the amendments and arguments presented.

The presented claims encompass bacterial strains comprising specific nucleotide sequences based upon their ammonia-oxidizing functions. Those strains must further have an amino acid sequence at least 96% identical to SEQ ID NO:1 and 2 and hybridizing to a specific probe, SEQ ID NO:5. The claims are thus not directed to an unrestricted amount of sequences based solely upon one factor, such as function. For example, a claim solely directed to a nucleotide sequence encoding a protein with the activity of ammonia-oxidation would not be

valid. The present claims have both structural and functional limitations. While some of the compounds having 96% homology might not be operative, *i.e.*, they might not be able to oxidize ammonia. However, in order to eliminate the inoperative embodiments, Applicants have coupled the requirement for maintaining a high degree of homology with SEQ ID NO:5, under specific stringent hybridization conditions, with the functional requirement that oxidation of ammonia must be maintained.

Structurally, the claims are limited to nucleotide sequences that are either identical in sequence to SEQ ID NO:1 and 2 or which are at least 96% homologous. Even though the claims therefore encompass a large number of specific sequences, the disclosure teaches one of skill in the art in screening for the required homology and testing for the claimed function (see for example, Examples 1-16). Thus, the homology requirement and functional requirement limit the number of compounds to a defined group, *i.e.*, the claims are not open-ended. *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 964 (Fed. Cir. 2002)(citing *Regents of the University of California v. Eli Lilly & Co.* 119 F.3d 1559 (Fed. Cir. 1997): According to the PTO, *Lilly* requires the disclosure of "detailed, relevant identifying characteristics ... i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics," and the Federal Circuit has adopted this interpretation.). Even though it would be impractical, one of ordinary skill in the art could, in principle, identify and write down every compound claimed. Thus, conceptually, Applicants were clearly in possession of the claimed invention at the time of filing.

In light of the above considerations, Applicants respectfully submit that the present claims meet the written description requirement of patentability. It is therefore respectfully requested that the Examiner's rejection of claims 15-21 based upon the written description requirement of patentability be withdrawn.

Conclusion

This response is being submitted within the three month deadline. In the case any fee is owed, please charge deposit account number 03-3975 (ref. 81289-284781). The Applicant believes that claims 1-7 and 15-21 are now in condition for allowance, and a favorable action is respectfully requested. If, for any reason, the Examiner finds the application other than in

condition for allowance, the Examiner is requested to call the undersigned attorney at the Los Angeles telephone number (213) 488-7100 to discuss the steps necessary for placing the application in condition for allowance should the Examiner believe that such a telephone conference would advance prosecution of the application.

Respectfully submitted,

PILLSBURY WINTHROP SHAW PITTMAN LLP

Date: 10/30/2006

By: _____
Carolyn S. Lu
Registration No. 56,817
Attorney For Applicant(s)

725 South Figueroa Street, Suite 2800
Los Angeles, CA 90017-5406
Telephone: (213) 488-7100
Facsimile: (213) 629-1033

Enclosures: Woese *et al.*, Proc. Nat'l. Academy Sci. 74(11): 5088-5090 (1977); Teske *et al.*, J. Bact. 176(21): 6623-6630 (1994).

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 5088–5090, November 1977
Evolution

Phylogenetic structure of the prokaryotic domain: The primary kingdoms

(archaebacteria/eubacteria/urkaryote/16S ribosomal RNA/molecular phylogeny)

CARL R. WOESE AND GEORGE E. FOX*

Department of Genetics and Development, University of Illinois, Urbana, Illinois 61801

Communicated by T. M. Sonneborn, August 18, 1977

ABSTRACT **A phylogenetic analysis based upon ribosomal RNA sequence characterization reveals that living systems represent one of three aboriginal lines of descent: (*i*) the eubacteria, comprising all typical bacteria; (*ii*) the archaebacteria, containing methanogenic bacteria; and (*iii*) the urkaryotes, now represented in the cytoplasmic component of eukaryotic cells.**

The biologist has customarily structured his world in terms of certain basic dichotomies. Classically, what was not plant was animal. The discovery that bacteria, which initially had been considered plants, resembled both plants and animals less than plants and animals resembled one another led to a reformulation of the issue in terms of a yet more basic dichotomy, that of eukaryote versus prokaryote. The striking differences between eukaryotic and prokaryotic cells have now been documented in endless molecular detail. As a result, it is generally taken for granted that all extant life must be of these two basic types.

Thus, it appears that the biologist has solved the problem of the primary phylogenetic groupings. However, this is not the case. Dividing the living world into *Prokaryotae* and *Eukaryotae* has served, if anything, to obscure the problem of what extant groupings represent the various primeval branches from the common line of descent. The reason is that eukaryote/prokaryote is not primarily a phylogenetic distinction, although it is generally treated so. The eukaryotic cell is organized in a different and more complex way than is the prokaryote; this probably reflects the former's composite origin as a symbiotic collection of various simpler organisms (1–5). However striking, these organizational dissimilarities do not guarantee that eukaryote and prokaryote represent phylogenetic extremes.

The eukaryotic cell *per se* cannot be directly compared to the prokaryote. The composite nature of the eukaryotic cell makes it necessary that it first be conceptually reduced to its phylogenetically separate components, which arose from ancestors that were noncomposite and so individually are comparable to prokaryotes. In other words, the question of the primary phylogenetic groupings must be formulated solely in terms of relationships among "prokaryotes"—i.e., noncomposite entities. (Note that in this context there is no suggestion *a priori* that the living world is structured in a dichotomous way.)

The organizational differences between prokaryote and eukaryote and the composite nature of the latter indicate an important property of the evolutionary process: Evolution seems to progress in a "quantized" fashion. One level or domain of organization gives rise ultimately to a higher (more complex) one. What "prokaryote" and "eukaryote" actually represent are two such domains. Thus, although it is useful to define phylogenetic patterns within each domain, it is not meaningful to construct phylogenetic classifications between domains: Prokaryotic kingdoms are not comparable to eukaryotic ones. This should be recognized by an appropriate terminology. The highest phylogenetic unit in the prokaryotic domain we think should be called an "urkingdom"—or perhaps "primary kingdom." This would recognize the qualitative distinction between prokaryotic and eukaryotic kingdoms and emphasize that the former have primary evolutionary status.

The passage from one domain to a higher one then becomes a central problem. Initially one would like to know whether this is a frequent or a rare (unique) evolutionary event. It is traditionally assumed—without evidence—that the eukaryotic domain has arisen but once; all extant eukaryotes stem from a common ancestor, itself eukaryotic (2). A similar prejudice holds for the prokaryotic domain (2). [We elsewhere argue (6) that a hypothetical domain of lower complexity, that of "progenotes," may have preceded and given rise to the prokaryotes.] The present communication is a discussion of recent findings that relate to the urkingdom structure of the prokaryotic domain and the question of its unique as opposed to multiple origin.

Phylogenetic relationships cannot be reliably established in terms of noncomparable properties (7). A comparative approach that can measure degree of difference in comparable structures is required. An organism's genome seems to be the ultimate record of its evolutionary history (8). Thus, comparative analysis of molecular sequences has become a powerful approach to determining evolutionary relationships (9, 10).

To determine relationships covering the entire spectrum of extant living systems, one optimally needs a molecule of appropriately broad distribution. None of the readily characterized proteins fits this requirement. However, ribosomal RNA does. It is a component of all self-replicating systems; it is readily isolated; and its sequence changes but slowly with time—permitting the detection of relatedness among very distant species (11–13). To date, the primary structure of the 16S (18S) ribosomal RNA has been characterized in a moderately large and varied collection of organisms and organelles, and the general phylogenetic structure of the prokaryotic domain is beginning to emerge.

A comparative analysis of these data, summarized in Table 1, shows that the organisms clearly cluster into several primary kingdoms. The first of these contains all of the typical bacteria so far characterized, including the genera *Acetobacterium, Acinetobacter, Acholeplasma, Aeromonas, Alcaligenes, Anacystis, Aphanocapsa, Bacillus, Bdellovibrio, Chlorobium, Chromatium, Clostridium, Corynebacterium, Escherichia, Eubacterium, Lactobacillus, Leptospira, Micrococcus, Mycoplasma, Paracoccus, Photobacterium, Propionibacterium,*

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Department of Biophysical Sciences, University of Houston, Houston, TX 77004.

Table 1. Association coefficients (S_{AB}) between representative members of the three primary kingdoms

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Saccharomyces cerevisiae*, 18S	—	0.29	0.33	0.05	0.06	0.08	0.09	0.11	0.08	0.11	0.11	0.08	0.08
2. *Lemna minor*, 18S	0.29	—	0.36	0.10	0.05	0.06	0.10	0.09	0.11	0.10	0.10	0.13	0.07
3. L cell, 18S	0.33	0.36	—	0.06	0.06	0.07	0.07	0.09	0.06	0.10	0.10	0.09	0.07
4. *Escherichia coli*	0.05	0.10	0.06	—	0.24	0.25	0.28	0.26	0.21	0.11	0.12	0.07	0.12
5. *Chlorobium vibrioforme*	0.06	0.05	0.06	0.24	—	0.22	0.22	0.20	0.19	0.06	0.07	0.06	0.09
6. *Bacillus firmus*	0.08	0.06	0.07	0.25	0.22	—	0.34	0.26	0.20	0.11	0.13	0.06	0.12
7. *Corynebacterium diphtheriae*	0.09	0.10	0.07	0.28	0.22	0.34	—	0.23	0.21	0.12	0.12	0.09	0.10
8. *Aphanocapsa* 6714	0.11	0.09	0.09	0.26	0.20	0.26	0.23	—	0.31	0.11	0.11	0.10	0.10
9. Chloroplast (*Lemna*)	0.08	0.11	0.06	0.21	0.19	0.20	0.21	0.31	—	0.14	0.12	0.10	0.12
10. *Methanobacterium thermoautotrophicum*	0.11	0.10	0.10	0.11	0.06	0.11	0.12	0.11	0.14	—	0.51	0.25	0.30
11. *M. ruminantium* strain M-1	0.11	0.10	0.10	0.12	0.07	0.13	0.12	0.11	0.12	0.51	—	0.25	0.24
12. *Methanobacterium* sp., Cariaco isolate JR-1	0.08	0.13	0.09	0.07	0.06	0.06	0.09	0.10	0.10	0.25	0.25	—	0.32
13. *Methanosarcina barkeri*	0.08	0.07	0.07	0.12	0.09	0.12	0.10	0.10	0.12	0.30	0.24	0.32	—

The 16S (18S) ribosomal RNA from the organisms (organelles) listed were digested with T1 RNase and the resulting digests were subjected to two-dimensional electrophoretic separation to produce an oligonucleotide fingerprint. The individual oligonucleotides on each fingerprint were then sequenced by established procedures (13, 14) to produce an oligonucleotide catalog characteristic of the given organism (3, 4, 13–17, 22, 23; unpublished data). Comparisons of all possible pairs of such catalogs defines a set of association coefficients (S_{AB}) given by: $S_{AB} = 2N_{AB}/(N_A + N_B)$, in which N_A, N_B, and N_{AB} are the total numbers of nucleotides in sequences of hexamers or larger in the catalog for organism A, in that for organism B, and in the interreaction of the two catalogs, respectively (13, 23).

Pseudomonas, *Rhodopseudomonas*, *Rhodospirillum*, *Spirochaeta*, *Spiroplasma*, *Streptococcus*, and *Vibrio* (refs. 13–17; unpublished data). The group has three major subdivisions, the blue-green bacteria and chloroplasts, the "Gram-positive" bacteria, and a broad "Gram-negative" subdivision (refs. 3, 4, 13–17; unpublished data). It is appropriate to call this urkingdom the *eubacteria*.

A second group is defined by the 18S rRNAs of the eukaryotic cytoplasm—animal, plant, fungal, and slime mold (unpublished data). It is uncertain what ancestral organism in the symbiosis that produced the eukaryotic cell this RNA represents. If there had been an "engulfing species" (1) in relation to which all the other organisms were endosymbionts, then it seems likely that 18S rRNA represents that species. This hypothetical group of organisms, in one sense the major ancestors of eukaryotic cells, might appropriately be called *urkaryotes*. Detailed study of anaerobic amoebae and the like (18), which seem not to contain mitochondria and in general are cytologically simpler than customary examples of eukaryotes, might help to resolve this question.

Eubacteria and urkaryotes correspond approximately to the conventional categories "prokaryote" and "eukaryote" when they are used in a phylogenetic sense. However, they do not constitute a dichotomy; they do not collectively exhaust the class of living systems. There exists a third kingdom which, to date, is represented solely by the methanogenic bacteria, a relatively unknown class of anaerobes that possess a unique metabolism based on the reduction of carbon dioxide to methane (19–21). *These "bacteria" appear to be no more related to typical bacteria than they are to eukaryotic cytoplasms.* Although the two divisions of this kingdom appear as remote from one another as blue-green algae are from other eubacteria, they nevertheless correspond to the *same* biochemical phenotype. The apparent antiquity of the methanogenic phenotype plus the fact that it seems well suited to the type of environment presumed to exist on earth 3–4 billion years ago lead us tentatively to name this urkingdom the *archaebacteria*. Whether or not other biochemically distinct phenotypes exist in this kingdom is clearly an important question upon which may turn our concept of the nature and ancestry of the first prokaryotes.

Table 1 shows the three urkingdoms to be equidistant from one another. Because the distances measured are actually proportional to numbers of mutations and not necessarily to time, it cannot be proven that the three lines of descent branched from the common ancestral line at about the same time. One of the three may represent a far earlier bifurcation than the other two, making there in effect only two urkingdoms. Of the three possible unequal branching patterns the case for which the initial bifurcation defines urkaryotes vs. all bacteria requires further comment because, as we have seen, there is a predilection to accept such a dichotomy.

The phenotype of the methanogens, although ostensibly "bacterial," on close scrutiny gives no indication of a specific phylogenetic resemblance to the eubacteria. For example, methanogens do have cell walls, but these do not contain peptidoglycan (24). The biochemistry of methane formation appears to involve totally unique coenzymes (23, 25, 26). The methanogen rRNAs are comparable in size to their eubacterial counterparts, but resemble the latter specifically in neither sequence (Table 1) nor in their pattern of base modification (23). The tRNAs from eubacteria and eukaryotes are characterized by a common modified sequence, TΨCG; methanogens modify this tRNA sequence in a quite different and unique way (23). It must be recognized that very little is known of the general biochemistry of the methanogens—and almost nothing is known regarding their molecular biology. Hence, although the above points are few in number, they represent most of what is now known. There is no reason at present to consider methanogens as any closer to eubacteria than to the "cytoplasmic component" of the eukaryote. Both in terms of rRNA sequence measurement and in terms of general phenotypic differences, then, the three groupings appear to be distinct urkingdoms.

If a third urkingdom exists, does this suggest that many more such will be found among yet to be characterized organisms? We think not, although the matter clearly requires an exhaustive search. As seen above, the number of species that can be classified as eubacteria is moderately large. To this list can be added *Spirillum* and *Desulfovibrio*, whose rRNAs appear typically eubacterial by nucleic acid hybridization measurements (27). Because the list is also phenotypically diverse, it seems unlikely that many, if any, of the yet uncharacterized

prokaryotic groups will be shown to have coequal status with the present three. Conceivably the halophiles whose cell walls contain no peptidoglycan, are candidates for this distinction (28, 29).

Eukaryotic organelles, however, could be a different matter. There can be no doubt that the chloroplast is of specific eubacterial origin (3, 4). A question arises with the remaining organelles and structures. Mitochondria, for example, do not conform well to a "typically prokaryotic" phenotype, which has led some to conclude that they could not have arisen as endosymbionts (30). By using "prokaryote" in a phylogenetic sense, this formulation of the issue does not recognize a third alternative—that the organelle in question arose endosymbiotically from a separate line of descent whose phenotype is not "typically prokaryotic" (i.e., eubacterial). It is thus conceivable that some endosymbiotically formed structures represent still other major phylogenetic groups; some could even be the only extant representation thereof.

The question that remains to be answered is whether the common ancestor of all three major lines of descent was itself a prokaryote. If not, each urkingdom represents an independent evolution of the prokaryotic level of organization. Obviously, much more needs to be known about the general properties of all the urkingdoms before this matter can be definitely settled. At present we can point to two arguments suggesting that each urkingdom does represent a separate evolution of the prokaryotic level of organization.

The first argument concerns the stability of the general phenotypes. The general eubacterial phenotype has been stable for at least 3 billion years—i.e., the apparent age of blue-green algae (31). The methanogenic phenotype seems to be at least this old in that branchings within the two urkingdoms are comparably deep (see Table 1). The time available to form each phenotype (from their common ancestor) is then short by comparison, which seems paradoxical in that the two phenotypes are so fundamentally different. We think that this ostensible paradox implies that the common ancestor in this case was not a prokaryote. It was a far simpler entity; it probably did not evolve at the "slow" rate characteristic of prokaryotes; it did not possess many of the features possessed by prokaryotes, and so these evolved independently and differently in separate lines of descent.

The second argument concerns the quality of the differences in the three general phenotypes. It seems highly unlikely, for example, that differences in general patterns of base modification in rRNAs and tRNAs are related to the niches that organisms occupy. Rather, differences of this nature imply independent evolution of the properties in question. It has been argued elsewhere that features such as RNA base modification generally represent the final stage in the evolution of translation (32). If these features have evolved separately in two lines of descent, their common ancestor, lacking them, had a more rudimentary version of the translation mechanism and consequently, could not have been as complex as a prokaryote (6).

With the identification and characterization of the urkingdoms we are for the first time beginning to see the overall phylogenetic structure of the living world. It is not structured in a bipartite way along the lines of the organizationally dissimilar prokaryote and eukaryote. Rather, it is (at least) tripartite, comprising (*i*) the typical bacteria, (*ii*) the line of descent manifested in eukaryotic cytoplasms, and (*iii*) a little explored grouping, represented so far only by methanogenic bacteria.

The ideas expressed herein stem from research supported by the National Aeronautics and Space Administration and the National Science Foundation. We are grateful to a number of colleagues who have helped to generate the yet unpublished data that make these speculations possible: William Balch, Richard Blakemore, Linda Bonen, Tristan Dyer, Jane Gibson, Ramesh Gupta, Robert Hespell, Bobby Joe Lewis, Kenneth Luehrsen, Linda Magrum, Jack Maniloff, Norman Pace, Mitchel Sogin, Stephan Sogin, David Stahl, Ralph Tanner, Thomas Walker, Ralph Wolfe, and Lawrence Zablen. We thank Linda Magrum and David Nanney for suggesting the name "archaebacteria."

1. Stanier, R. Y. (1970) *Symp. Soc. Gen. Microbiol.* **20**, 1–38.
2. Margulis, L. (1970) *Origin of Eucaryotic Cells* (Yale University Press, New Haven).
3. Zablen, L. B., Kissel, M. S., Woese, C. R. & Buetow, D. E. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2418–2422.
4. Bonen, L. & Doolittle, W. F. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2310–2314.
5. Bonen, L., Cunningham, R. S., Gray, M. W. & Doolittle, W. F. (1977) *Nucleic Acid Res.* **4**, 663–671.
6. Woese, C. R. & Fox, G. E. (1977) *J. Mol. Evol.*, in press.
7. Sneath, P. H. A. & Sokal, R. R. (1973) *Numerical Taxonomy* (W. H. Freeman, San Francisco).
8. Zuckerkandl, E. & Pauling, L. (1965) *J. Theor. Biol.* **8**, 357–366.
9. Fitch, W. M. & Margoliash, E. (1967) *Science* **155**, 279–284.
10. Fitch, W. M. (1976) *J. Mol. Evol.* **8**, 13–40.
11. Sogin, S. J., Sogin, M. L. & Woese, C. R. (1972) *J. Mol. Evol.* **1**, 173–184.
12. Woese, C. R., Fox, G. E., Zablen, L., Uchida, T., Bonen, L., Pechman, K., Lewis, B. J. & Stahl, D. (1975) *Nature* **254**, 83–86.
13. Fox, G. E., Pechman, K. R. & Woese, C. R. (1977) *Int. J. Syst. Bacteriol.* **27**, 44–57.
14. Uchida, T., Bonen, L., Schaup, H. W., Lewis, B. J., Zablen, L. B. & Woese, C. R. (1974) *J. Mol. Evol.* **3**, 63–77.
15. Zablen, L. B. & Woese, C. R. (1975) *J. Mol. Evol.* **5**, 25–34.
16. Doolittle, W. F., Woese, C. R., Sogin, M. L., Bonen, L. & Stahl, D. (1975) *J. Mol. Evol.* **4**, 307–315.
17. Pechman, K. J., Lewis, B. J. & Woese, C. R. (1976) *Int. J. Syst. Bacteriol.* **26**, 305–310.
18. Bovee, E. C. & Jahn, T. L. (1973) in *The Biology of Amoeba*, ed. Jeon, K. W. (Academic Press, New York), p. 38.
19. Wolfe, R. S. (1972) *Adv. Microbiol. Phys.* **6**, 107–146.
20. Zeikus, J. G. (1977) *Bacteriol. Rev.* **41**, 514–541.
21. Zeikus, J. G. & Bowen, V. G. (1975) *Can. J. Microbiol.* **21**, 121–129.
22. Balch, W. E., Magrum, L. J., Fox, G. E., Wolfe, R. S. & Woese, C. R. (1977) *J. Mol. Evol.*, in press.
23. Fox, G. E., Magrum, L. J., Balch, W. E., Wolfe, R. S. & Woese, C. R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4537–4541.
24. Kandler, O. & Hippe, H. (1977) *Arch. Microbiol.* **113**, 57–60.
25. Taylor, C. D. & Wolfe, R. S. (1974) *J. Biol. Chem.* **249**, 4879–4885.
26. Cheeseman, P., Toms-Wood, A. & Wolfe, R. S. (1972) *J. Bacteriol.* **112**, 527–531.
27. Pace, B. & Campbell, L. L. (1971) *J. Bacteriol.* **107**, 543–547.
28. Brown, A. D. & Cho, K. Y. (1970) *J. Gen. Microbiol.* **62**, 267–270.
29. Reistad, R. (1972) *Arch. Mikrobiol.* **82**, 24–30.
30. Raff, R. A. & Mahler, H. R. (1973) *Science* **180**, 517–521.
31. Shopf, J. W. (1972) *Exobiology—Frontiers of Biology* (North Holland, Amsterdam), Vol. 23, pp. 16–61.
32. Woese, C. R. (1970) *Symp. Soc. Gen. Microbiol.* **20**, 39–54.

JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 6623–6630
0021-9193/94/$04.00+0

Vol. 176, No. 21

Evolutionary Relationships among Ammonia- and Nitrite-Oxidizing Bacteria

A. TESKE,[1]† E. ALM,[1] J. M. REGAN,[2]‡ S. TOZE,[1]§ B. E. RITTMANN,[2]‖ AND D. A. STAHL[1,2,3]*

Department of Veterinary Pathobiology,[1] Department of Civil Engineering,[2] and Department of Microbiology,[3] University of Illinois, Urbana, Illinois 61801

Received 5 May 1994/Accepted 22 August 1994

Comparative 16S rRNA sequencing was used to evaluate phylogenetic relationships among selected strains of ammonia- and nitrite-oxidizing bacteria. All characterized strains were shown to be affiliated with the proteobacteria. The study extended recent 16S rRNA-based studies of phylogenetic diversity among nitrifiers by the comparison of eight strains of the genus *Nitrobacter* and representatives of the genera *Nitrospira* and *Nitrospina*. The later genera were shown to be affiliated with the delta subdivision of the proteobacteria but did not share a specific relationship to each other or to other members of the delta subdivision. All characterized *Nitrobacter* strains constituted a closely related assemblage within the alpha subdivision of the proteobacteria. As previously observed, all ammonia-oxidizing genera except *Nitrosococcus oceanus* constitute a monophyletic assemblage within the beta subdivision of the proteobacteria. Errors in the 16S rRNA sequences for two strains previously deposited in the databases by other investigators (*Nitrosolobus multiformis* C-71 and *Nitrospira briensis* C-128) were corrected. Consideration of physiology and phylogenetic distribution suggested that nitrite-oxidizing bacteria of the alpha and gamma subdivisions are derived from immediate photosynthetic ancestry. Each nitrifier retains the general structural features of the specific ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, apparently are not derived from an ancestral nitrifying phenotype.

Biologists have been asking questions concerning the evolutionary origins of, and phylogenetic relationships among, chemolithotrophic microorganisms for well over a century. However, it has been only in the last decade that comparative molecular studies have provided the basis to shape an understanding of their phylogeny. Most notably, the use of comparative rRNA sequencing has provided an all-encompassing phylogenetic framework within which all the chemolithotrophs can be placed. The emerging phylogeny has, in turn, provided insights into their antiquity and the origins of lithotrophic metabolism. For example, sulfur oxidation and iron oxidation appear to be evolutionarily early and widespread metabolic modes that are not confined to a single phylogenetic assemblage of bacteria (27). An important group of chemolithotrophs commonly called nitrifying bacteria or nitrifiers, the nitrite- and ammonia-oxidizing bacteria, has to be reconsidered as well.

These classical chemolithotrophs are still viewed as one coherent group, the family *Nitrobacteriaceae* (45, 46), defined by their characteristic ability to grow as lithotrophs by oxidation of ammonia to nitrite or nitrite to nitrate. No organism that has been described is capable of fully oxidizing ammonia to nitrate. Consequently, the classification of nitrifying bacteria is based primarily upon oxidation of either ammonia or nitrite, with secondary consideration of cell shape and the highly characteristic cytoplasmic membrane structures (46). All known ammonia-oxidizing bacteria are obligate chemolithoautotrophs. In contrast, some nitrite-oxidizing bacteria are mixotrophs and also can grow heterotrophically (4, 26). Although the existing taxonomy assigns these bacteria to a single family, accumulating biochemical and molecular data do not support their phylogenetic coherence.

Physiological and enzymatic data argue against a close relatedness between ammonia and nitrite oxidizers. They employ two very different key enzyme systems for the energy-gaining oxidation of ammonia and nitrite (5). Comparative sequencing studies based on 16S rRNA oligonucleotide cataloging provided the first outline of phylogenetic diversity of nitrifying bacteria (56, 56a, 57). More recently, the phylogeny of the ammonia-oxidizing bacteria has been refined on the basis of near-complete 16S rRNA sequences (17). These results are consistent with the earlier cataloging studies. However, this picture is far from complete. Many new species were not included in sequencing studies. Others have been characterized by DNA-DNA hybridization and GC content but not by sequence comparisons.

In this study, we used comparative 16S rRNA sequencing to further delineate and refine the phylogeny of ammonia- and nitrite-oxidizing bacteria. In so doing, we identify closely related nonnitrifying species and discuss the physiological and evolutionary significances of their recent common origins.

MATERIALS AND METHODS

Bacterial strains and 16S rRNA sequences. Strains sequenced in this study are listed in Table 1. Nitrifier species and strains were obtained from the American Type Culture Collection and from J. B. Waterbury, Woods Hole Marine Biology Laboratory, Woods Hole, Mass., and J. Prosser, University of Aberdeen, Aberdeen, Scotland. In addition to those sequences

* Corresponding author. Present address: Department of Civil Engineering, Northwestern University, 2145 Sheridan Rd., Evanston, IL 60208-3109. Phone: (708) 491-4997. Fax: (708) 491-4011. Electronic mail address: d-stahl@nwu.edu.

† Present address: Max Planck Institute for Marine Microbiology, D-28359, Bremen, Germany.

‡ Present address: Warzyn, Inc., Madison, WI 53705.

§ Present address: CSIRO Division of Water Resources, Perth, Western Australia, Australia 6014.

‖ Present address: Department of Civil Engineering, Northwestern University, Evanston, IL 60208-3109.

TABLE 1. Sources of bacterial strains used in this study

Species	Strain
Nitrobacter winogradskyi	ATCC 25381 type strain (46)
	ATCC 14123 (formerly designated *Nitrobacter agilis*, reassigned to the species *Nitrobacter winogradskyi* [20, 49])
Nitrobacter hamburgensis	X14 type strain (7) (from S. W. Watson and F. Valois)
Nitrobacter species	R6 (Rothamsted, United Kingdom, from J. Prosser)
Nitrobacter species[a]	ATCC 25383
Nitrobacter species[a]	ATCC 25384
Nitrobacter species[a]	ATCC 25385
Nitrococcus mobilis	ATCC 25380 type strain (46)
Nitrosolobus multiformis	ATCC 25196 type strain (46)
	ATCC 25198[a]
Nitrosomonas europae	ATCC 19718
Nitrosospira briensis	C-128 (from S. W. Watson and F. Valois)
Nitrospina gracilis	Nb-3, Pacific Ocean isolate (from S. W. Watson and F. Valois)
	Nb-211, Atlantic Ocean isolate (from S. W. Watson and F. Valois)
Nitrospira marina	Nb-295 (from S. W. Watson and F. Valois)

[a] Sequence determined by reverse transcriptase sequencing.

determined in this study, previously determined 16S rRNA sequences used for comparison were obtained from the EMBL and RDP databases (29). Unpublished 16S rRNA sequences or sequences previously determined for nitrifying species (and corresponding accession numbers) are those recently reported by Head et al. (17) (*Nitrosospira briensis* strain C-128 [M96396], *Nitrosovibrio tenuis* strain C-141 [M96397], *Nitrosococcus oceanus* strain C-27 [M96398], *Nitrosomonas europaea* strain C-31 [M96399], *Nitrosomonas marina* strain C-56 [M96400], *Nitrosolobus multiformis* strain C-71 [M96401], *Nitrosomonas eutropha* strain C-91 [M96402], *Nitrococcus mobilis* strain Nc2 type strain [M96403], and *Nitrosovibrio tenuis* strain Nv12 [M96405]) and those available from the RDP (29) (*Nitrosomonas europaea* and *Nitrosolobus multiformis*).

Isolation of nucleic acids and sequencing. Nucleic acids were isolated by mechanical disruption of cells and phenol extraction as previously described (40). The 16S rRNA was isolated and sequenced directly, using the reverse transcriptase method of Lane et al. (28). DNA sequencing was done by using PCR and direct sequencing of PCR products. For direct sequencing, 16S rRNA genes were amplified by using two primers, 11F (5'-GTTTGATCCTGGCTCAG-3', corresponding to *Escherichia coli* positions 11 to 27) and 1512-AR (5'-ACGGT/CTACCTTGTTACGACTT-3', corresponding to *E. coli* positions 1492 to 1513). Each of the 35 cycles started with 1 min of denaturation at 95°C, continued with 2 min of annealing at 40°C, and ended with 3 min of elongation at 71°C. The reaction mix (total volume, 50 or 100 μl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM (each) dGTP, dATP, dTTP, and dCTP, 0.5 to 1.0 U of *Taq* DNA polymerase (United States Biochemical Co., Cleveland, Ohio), and 1 μM (each) primer.

The PCR products were analyzed by electrophoresis on 1% horizontal agarose gels in TAE buffer (36), using a 1-kb DNA ladder as a size marker (Difco, Detroit, Mich.; Gibco BRL, Grand Island, N.Y.). The DNA from the region of the gel containing the PCR amplification product of appropriate size was electrophoretically recovered by cutting a small well in the gel directly in front of the selected PCR product. Electrophoresis was continued until the DNA migrated into the buffer-filled well. The buffer was quickly transferred to a microcentrifuge tube, and 0.1 volume of 5 M NaCl and 2.5 volumes of ethanol were added. Following incubation for 1 h at −80°C, the DNA was pelleted by centrifugation and directly used in the sequencing reactions (9), using primers complementary to highly conserved regions of 16S rRNA sequence. The previously published protocol of Böttger (9) was modified to include addition of 1 μl of aqueous 5% (wt/vol) Nonidet P-40 (Sigma Co., St. Louis, Mo.) to the template-primer mix (0.5% in a final reaction volume of 10 μl). In addition, denaturation at 94°C was extended to 10 min and the 37°C renaturation step was omitted.

The sequencing primers and corresponding positions within the *E. coli* 16S rRNA were 11F (5'-GTTTGATCCTGGCTCAG-3' [11 to 27]), 61F (5'-GCTTAACACATGCAAG-3' [46 to 61]), 343aR (5'CTGCTGCCTCCCGTA-3' [357 to 341]), 519R (5'-GWATTACCGCGGCKGCTG-3' [536 to 519]), 530F (5'-GTGCCAGC[A/C]GCCGCGG-3' [515 to 530]), 690R (5'-TCTACGCATTTCACC-3' [704 to 690]), 786R (5'-CTACT[C/G]GGGTATCTAATC-3' [803 to 786]), 802F (5'-ATTAGATACCCTGGTA-3' [787 to 802]), 922F (5'-GAAACTTAAA[G/T]GAATTG-3' [906 to 922]), 956R (5'-GGCGTTGTGTC[C/G]AATTAA-3' [974 to 956]), 1056R (5'-ACGAGCTGACGAC[A/G]GCCA-3' [1073 to 1056]), 1114F (5'-GCAACGAGCGCAACCC-3' [1099 to 1114]), 1100R (5'-AGGGTTGCGCTCGTTG-3' [1115 to 1100]), 1240F (5'-ACACGCGTGCTACAAT-3' [1225 to 1240]), 1227R (5'-CCATTGTAGCACGTGT-3' [1242 to 1227]), 1406F (5'-TG[C/T]ACACACCGCCCGT-3' [1391 to 1406]), 1392R (5'-ACGGGCGGTGTGT[G/A]C-3' [1406 to 1392]), and 1512-AR (5'-ACGG[T/C]TACCTTGTTACGACTT-3' [1512 to 1492]).

Alignment and phylogenetic tree inference. The sequences were aligned by secondary structure according to the RDP database alignment (29). The SIMILARITY_RANK tool of the RDP database was used to search the RDP database for close evolutionary relatives of different nitrifier sequences. Phylogenetic trees were inferred, using the PAUP3.1 parsimony program package (42a) and the distance matrix method of Fitch and Margoliash as implemented by PHYLIP (Phylogeny Inference Package) version 3.5c (15). Regions of ambiguous sequence alignment were excluded from analysis.

Nucleotide sequence accession numbers. Sequences have been deposited in GenBank under accession numbers L35501 to L35514.

RESULTS AND DISCUSSION

The now recognized diversity of genera and species of the ammonia oxidizers includes *Nitrosomonas*, with at least 10 species (19, 23, 53), *Nitrosococcus* (53), with 3 species (22, 25, 53), *Nitrosospira*, with 1 recognized species (44, 54) and 4 other species indicated by DNA homology studies (24), *Nitrosovibrio*, with 1 described species (16) and 1 additional species indicated by DNA homology studies (24), and *Nitrosolobus*, with one described species (49) and 1 additional species indicated by DNA homology studies (24). The nitrite-oxidizing genera have received relatively less attention, and few have been examined by comparative sequence analysis. The four recognized genera of nitrite-oxidizing bacteria include *Nitrobacter* (53), with three species (6, 7), and *Nitrospina* (50), *Nitrococcus* (50), and *Nitrospira* (47), with one species each.

Previous comparative studies and the sequence data presented here indicate that all characterized nitrifying bacteria are members of the proteobacteria, a large bacterial group of presumed photosynthetic ancestry (55, 56). Relationships

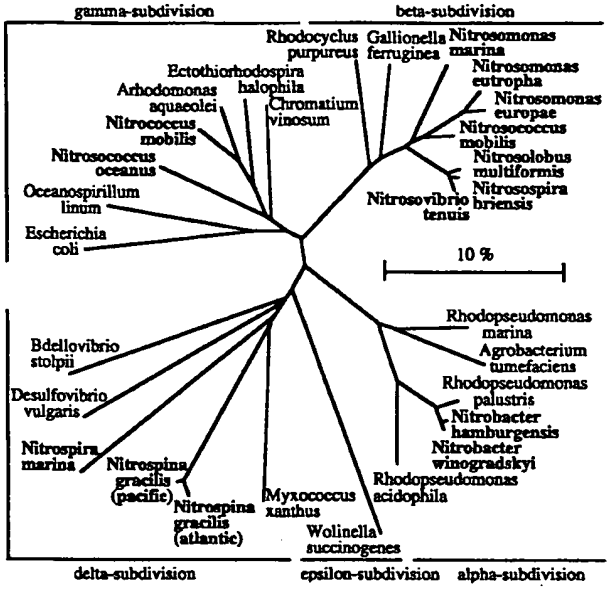

FIG. 1. Distance tree for the the proteobacteria, including the nitrifying isolates characterized in this study. The scale bar corresponds to 0.1 estimated fixed mutation per sequence position.

within the proteobacteria as inferred by comparative 16S rRNA sequence analyses are displayed in Fig. 1. The general phylogenetic distribution is briefly described here and discussed in greater detail in the following sections, which elaborate specific affiliations between nitrifying and nonnitrifying representatives of the individual proteobacterial subdivisions.

Nitrite-oxidizing genera occur in the alpha, gamma, and delta subdivisions of the proteobacteria. The genus *Nitrobacter* forms a tight group of very closely related species in the alpha subdivision, clustering together with species of the genera *Bradyrhizobium*, *Rhodopseudomonas*, and *Afipia*. *Nitrococcus mobilis* is a member of the gamma subdivision, related to members of the family *Ectothiorhodospiraceae* and the ammonia-oxidizing bacterium *Nitrosococcus oceanus*. *Nitrospira marina*, the only described species of the genus, shows an affiliation with the delta subdivision. The two strains of *Nitrospina gracilis* (the only described species in the genus) also demonstrate a peripheral relationship to the delta subdivision but are unrelated to *Nitrospira marina*. The ammonia-oxidizing bacteria (with the exception of *Nitrosococcus oceanus*) constitute a closely related assemblage within the beta subdivision composed of the genera *Nitrosolobus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosomonas*. In contrast, the two species of the genus *Nitrosococcus* belong to different subdivisions (17, 55). *Nitrosococcus mobilis* is related to the genus *Nitrosomonas* in the beta subdivision; *Nitrosococcus oceanus* is the only recognized ammonia oxidizer in the gamma subdivision.

For a more detailed comparison of relationships, we consider each subdivision separately. This treatment is a consequence of the requirement for sequence alignment prior to phylogenetic analysis. By comparing only closely related sequences, to the exclusion of sequences from different subdivisions, it is possible to include a greater number of aligned sequence positions in the comparative analyses. Highly variable regions of sequence generally must be excluded when distantly related sequences are compared, because they cannot be unambiguously aligned. A similar treatment was recently used to characterize sulfur- and iron-oxidizing bacteria (27). The following discussion is based on an independent analysis of each of the four subdivisions.

The alpha subdivision: the genus *Nitrobacter*. The members of the genus *Nitrobacter* make up an exclusive and highly related cluster that is closely associated with *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, and *Afipia clevelandensis* within the alpha-2 branch of the proteobacteria (Fig. 2). More distantly related sequences include those of an additional photosynthetic species (e.g., *Rhodopseudomonas acidophila*) and methylotrophs.

Near-complete sequences were determined for four *Nitrobacter* strains: *Nitrobacter winogradskyi* ATCC 14123 (formerly *Nitrobacter agilis*), *Nitrobacter winogradskyi* ATCC 25381, *Nitrobacter hamburgensis* X14, and *Nitrobacter* sp. strain R6 (isolated by J. Prosser, Rothamsted, United Kingdom). The

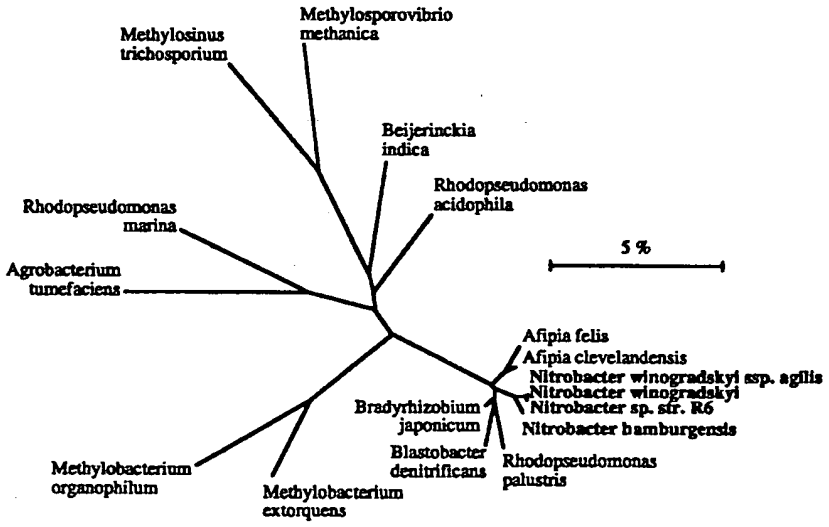

FIG. 2. Distance tree for the alpha subdivision of the proteobacteria.

16S rRNA sequences for the two strains of *Nitrobacter winogradskyi* (ATCC 14123 and ATCC 25381) were identical and differed from *Nitrobacter* sp. strain R6 at only one nucleotide position (*E. coli* position 1267, C/U). *Nitrobacter hamburgensis* X14 differs in at least nine nucleotide positions from these three strains. In addition to the near-complete sequences determined for these members of the genus, partial sequences of three additional *Nitrobacter* strains (ATCC 25383, ATCC 25384, and ATCC 25385) were determined by rRNA-templated sequencing with reverse transcriptase. Approximately 500 nucleotides of 16S rRNA sequence beginning at the 5′ end were determined for each. The partial sequences of strain 25383 and 25384, not considering ambiguous nucleotide assignments, are identical to the near-complete sequences determined for *Nitrobacter winogradskyi* strains ATCC 25381 and 14123. However, *Nitrobacter* sp. strain 25383 differs at two nucleotide positions, *E. coli* positions 250 (A/U) and 264 (C/U). Thus, additional genetic diversity exists among available *Nitrobacter* species and may be great enough to warrant species distinction.

Within the genus *Nitrobacter*, the pairwise evolutionary distance estimates do not exceed 1%, indicating a low degree of genetic diversity. These results are consistent with the recent 16S rRNA sequence comparisons of three *Nitrobacter* isolates (*N. winogradskyi*, *Nitrobacter* sp. strain LL, and *N. hamburgensis*) by Orso et al. (34). Many other genera of bacteria show 10 to 15% 16S rRNA sequence divergence between species, although this may be considered the upper limit for genus rank and more appropriate for the definition of families (14, 37). The high degree of genetic homogeneity within the genus *Nitrobacter* was also demonstrated by an earlier 16S rRNA oligonucleotide study (38) reporting a high S_{ab} (similarity) value (0.82) between *Nitrobacter winogradskyi* and *Nitrobacter hamburgensis*. However, we do not advocate the use of rRNA sequence divergence alone for defining taxonomic rank. This information must be integrated with phenotypic variation and environmental distribution.

The phylogenetic relationships between *Nitrobacter* species were also investigated by Navarro et al. (31). On the basis of restriction fragment length polymorphism data of PCR-amplified intergenic spacer regions of the ribosomal operon, they estimated sequence divergences of intergenic spacer regions in 39 different *Nitrobacter* strains. Their results are congruent with our 16S rRNA-based phylogeny of the genus *Nitrobacter*. The highest spacer region divergences (7.5 to 7.7%) were observed between *Nitrobacter hamburgensis* X14 and *Nitrobacter winogradskyi* ATCC 14123. Lower sequence divergences, in the range of 3 to 4%, were observed between *Nitrobacter winogradskyi* ATCC 14123 and various other *Nitrobacter* spp., including strain R6. DNA-DNA reassociation studies (7) demonstrated an overall DNA homology of 36% between species, in the accepted range (20 to 60%) for species (genospecies) relationship within a genus. Thus, the 16S rRNA provides information of more general relationships at approximately the species level of discrimination, whereas DNA homology and comparison of intergenic spacer regions provide resolution of individual strains.

The tight cluster of *Nitrobacter* species and several nonnitrifying species (*Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, and *Afipia clevelandensis*) was only partially outlined by earlier studies. Oligonucleotide data demonstrated a close relationship between the 16S rRNAs of *Nitrobacter winogradskyi* and *Nitrobacter hamburgensis* to that of *Rhodopseudomonas palustris* (38). A 16S rRNA sequence analysis of *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, and *Afipia clevelandensis* and

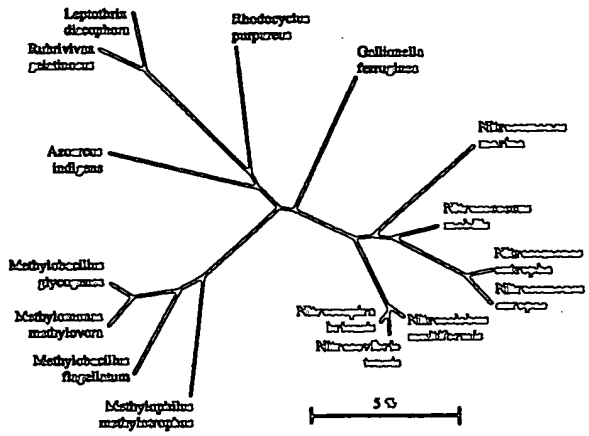

FIG. 3. Distance tree for the beta subdivision of the proteobacteria.

Afipia felis showed their close relationships (32, 52). A chemotaxonomic study (3, 30a), based on analysis of lipid A and deep core regions of cell wall lipopolysaccharides, was consistent with the sequence data presented here. The 16S rRNA sequences of *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, and *Nitrobacter winogradskyi* form a coherent cluster, well separated from other members of the alpha subdivision (96.8 to 100% sequence similarity). Thus, the root-nodulating *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, the human pathogens *Afipia felis* and *Afipia clevelandensis*, the anaerobic photosynthetic *Rhodopseudomonas palustris*, and the facultative lithoautotrophic *Nitrobacter* species all have a relatively recent common ancestor, also suggesting they have common traits. For example, the capacity for denitrification is present in *Blastobacter denitrificans*, *Nitrobacter* species, and some strains of *Rhodopseudomonas palustris* (8, 21, 43). However, nitrification has not been reported for *Blastobacter denitrificans* (43), and the need for a systematic comparison of these enzyme systems is indicated. Three members of this assemblage divide by budding, *Rhodopseudomonas palustris*, *Blastobacter denitrificans*, and *Nitrobacter* species (35, 41, 46). Thus, the other members of this assemblage should be reexamined for this characteristic. Another common phenotypic theme of related bacteria is the intracellular habitat of *Bradyrhizobium japonicum* and *Afipia* species (agents of cat scratch disease) (32). However, facultative or obligate intracellular associations are observed in a variety of the alpha-subdivision bacteria, including species of the genera *Rickettsia*, *Rochalimaea*, and *Brucella* (55), and so should not be considered unique to the specific nitrobacter lineage.

Related but physiologically distinct bacteria include the methylotrophs. Methylotrophs are affiliated with the alpha, beta, and gamma subdivisions of the proteobacteria in close peripheral relationship to both nitrite and ammonia oxidizers (Fig. 2 to 4). Within the alpha subdivision, *Methylobacterium* species are the closest methylotrophic relatives of the characterized *Nitrobacter* species. They have similar intracytoplasmic membrane structures, a feature that ties them both to the phototrophs (13). A relationship between methane-oxidizing and ammonia-oxidizing species was earlier suggested by the capacity of ammonia monooxygenase and methane monooxygenase to oxidize either ammonia or methane, but the evolutionary and ecophysiological significances of this oxidative flexibility are unresolved (18, 33). Nitrite-oxidizing genera,

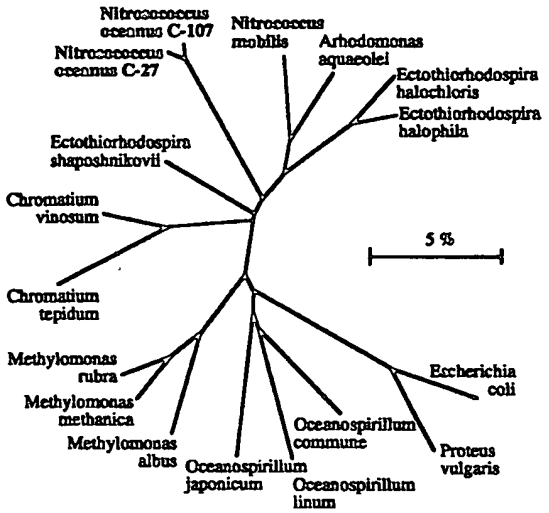

FIG. 4. Distance tree for the gamma subdivision of the proteobacteria.

such as *Nitrobacter*, *Nitrospina*, and *Nitrococcus* species, cannot oxidize methane (18). We call attention to similar peripheral affiliations of methylotrophs with nitrifiers in the beta and gamma subdivisions but will not further address these relationships.

The beta subdivision: the genera *Nitrosolobus*, *Nitrosospira*, *Nitrosovibrio*, *Nitrosomonas*, and *Nitrosococcus*. The physiological differences of ammonia-oxidizing and nitrite-oxidizing bacteria are reflected by their mutually exclusive phylogenetic positions as inferred by 16S rRNA sequence comparisons. However, since these sequences were independently reported, we restrict our discussion to general features of relationship between ammonia- and nitrite-oxidizing species and, when appropriate, differences in the independently determined sequences. The ammonia-oxidizing bacteria, with one exception (*Nitrosococcus oceanus*), constitute a monophyletic line of descent within the beta subdivision of the proteobacteria (98% bootstrap). No described nitrite oxidizer has been placed in this subdivision. Previous 16S rRNA oligonucleotide data (57) indicated a high degree of genetic homogeneity among the ammonia oxidizers, and this is now confirmed by analysis of near-complete 16S rRNA sequences (17). A recent taxonomic treatment of the genus *Nitrosomonas* by Koops et al. (23) classified eight new species of *Nitrosomonas*, including *Nitrosomonas marina*. The 5'-terminal sequence (ca. 500 nucleotides) of *Nitrosomonas europaea* ATCC 19718 is identical to that of strain C-31 (ATCC 25984). With reference to the phylogenetic relationships defined in Fig. 1 and 3, *Nitrosococcus mobilis* is encompassed by the now described genus *Nitrosomonas*. We suggest that taxonomic revision is necessary to include this additional information of phylogenetic relationship.

The closest relatives to the beta-subdivision ammonia oxidizers are the iron-oxidizing bacterium *Gallionella ferruginea*, the photosynthetic *Rhodocyclus purpureus*, and methylotrophic bacteria (Fig. 3). The close relationship to the chemolithotrophic *Gallionella ferruginea* is also reflected by an extensive intracellular membrane system, also found in some *Nitrosomonas* species, consisting (in part) of irregular tubes protruding into the cytoplasm continuous with the cytoplasmic membrane (30). The tubular membrane system of *R. purpureus* also resembles those of *Gallionella ferruginea* and some *Nitrosomonas* species.

Nitrosospira briensis (strain C-128) is one representative of at least two, and probably five, different genospecies. Two groups of *Nitrosospira* species could be distinguished on the basis of their G+C contents (24). DNA hybridization data allowed a further categorization into five species (24). However, supporting 16S rRNA sequence data are not yet available. In this regard, there are 10 nucleotide differences between the sequence of strain C-128 determined in this study and the previously published sequence for this strain (17). Every mismatch was checked and resequenced, confirming the sequence presented here. The character of the differences suggests that the differences arose from sequencing errors in the previously published sequence rather than strain confusion. Thus, this genus is currently represented by only one 16S rRNA sequence.

Nitrosolobus multiformis C-71 (ATCC 25196, type strain) represents one of two species defined by G+C content and DNA hybridization (24, 48). The 16S rRNA sequence of C-71 was determined in this study and independently by Larsen et al. (29). These sequences are identical at all comparable positions; however, the previously published sequence (17) differs at six nucleotide positions. These differences should be noted for future determinative and taxonomic studies. In addition, partial sequencing of the 5' termini (ca. 300 nucleotides) of *N. multiformis* ATCC 25198 revealed two differences at *E. coli* positions 122 (A/G) and 187 (C/U). However, additional sequencing is needed to determine the need for taxonomic revision.

A recent phylogenetic treatment of the ammonia oxidizers based on 16S rRNA sequence comparisons (17) recommended that the genera *Nitrosovibrio*, *Nitrosospira*, and *Nitrosolobus* be combined in a single genus. Although we concur that these genera constitute a closely related assemblage, of lesser phylogenetic depth than the described species of *Nitrosomonas*, we note that the ultrastructure of *Nitrosolobus* (cytomembranes that partially compartmentalize the cell) is distinct from those of *Nitrosospira* and *Nitrosovibrio* (both lacking an extensive cytomembrane system) (46). Thus, although *Nitrosovibrio* and *Nitrosospira* spp. could be accommodated within a single genus, we recommend reconsideration of inclusion of *Nitrosolobus* in such a revised taxonomic description.

The gamma subdivision: the genera *Nitrosococcus* and *Nitrococcus*. The gamma subdivision of the proteobacteria harbors the ammonia oxidizer *Nitrosococcus oceanus* (strains C-107 and C-27) and the nitrite oxidizer *Nitrococcus mobilis* (ATCC 25380, type strain). This is the only example of both metabolic types occurring within the same subdivision (Fig. 1 and 4). Both species are found within the purple sulfur bacterial line of descent.

Physiologically, the purple sulfur bacteria are divided between those depositing elemental sulfur inside the cell (*Chromatiaceae*) and those depositing sulfur outside (*Ectothiorhodospiraceae*). This division corresponds to phylogenetic relationships as shown earlier by oligonucleotide comparisons (39). *Nitrosococcus oceanus* and *Nitrococcus mobilis* are both members of the ectothiorhodospira branch. These associations are reminiscent of those seen in the alpha and beta subdivisions and again suggest that nitrifying bacteria are derived from photosynthetic ancestry. Similarly, the intracytoplasmic membrane system of *Nitrosococcus oceanus* resembles the stacked ectothiorhodospira type: several stacks in ectothiorhodospira and one central stack in *Nitrosococcus oceanus*. *Nitrococcus mobilis* differs in having tubes randomly arranged in the cytoplasm. Another similarity among them is common marine

origin. *Nitrococcus mobilis*, *Nitrosococcus oceanus*, and nearly all *Ectothiorhodospira* species require specific salt conditions for growth. The closest relative of *Nitrococcus mobilis* is a bacterium within the ectothiorhodospira lineage, *Arhodomonas aquaeolei*, a recently described, aerobic, halophilic bacterium isolated from subterranean brine (1). The relatively close relationship between *Nitrosococcus oceanus* and *Nitrococcus mobilis* raises the question of independent or derived origins of the corresponding ammonia- and nitrite-oxidizing enzyme systems.

The delta subdivision: the genera *Nitrospina* and *Nitrospira*. There were no precedents for assigning phylogenetic affiliations of the genera *Nitrospina* and *Nitrospira*. The 16S sequence of *Nitrospira marina* strain Nb-295 marks an early divergence within the delta subdivision of the proteobacteria. The two strains of *Nitrospina gracilis*, Nb-211 (Atlantic strain) and Nb-3 (Pacific strain), are closely related and define a second early divergence within the delta subdivision. Neither genus is closely related to any known member of the delta proteobacteria (e.g., *Myxococcus xanthus* or *Bdellovibrio bacteriovorans*) (Fig. 1).

The affiliation of *Nitrospina* and *Nitrospira* with the delta subdivision is also supported by specific nucleotide signatures (55). *Nitrospina gracilis* and *Nitrospira marina* share the sequence motif CCTGACGCAGC(G/A)ACGCCG (*E. coli* 16S rRNA positions 385 to 402) common to all delta-subgroup sulfate reducers and also *M. xanthus* (2). Their distance from *Nitrobacter* species (alpha subdivision) and *Nitrococcus mobilis* (gamma subdivision) is reflected by different nitrite-oxidizing enzymes; none of the five major protein bands of the nitrite-oxidizing membrane of *Nitrobacter hamburgensis* (42) was detected in *Nitrospira marina* (47).

In contrast to the other subdivisions, the delta subdivision contains no known phototrophs. The ancestor of this subdivision is assumed to have lost its photosynthetic ability (55). The *Nitrospina* and *Nitrospira* species probably represent a type of nitrifying metabolism that is not derived directly from immediate photosynthetic ancestry. Consistent with this view, the nitrite-oxidizing intracytoplasmic membranes common to other subdivision genera, thought to be derived from photosynthetic precursors, are lacking in *Nitrospina* and *Nitrospira* species.

Summary. *Nitrobacteriaceae* is a polyphyletic family. Nitrite- and ammonia-oxidizing bacteria are widely distributed within the proteobacteria, demonstrating a phylogenetic diversity comparable to that previously reported for photosynthetic bacteria and sulfur- and iron-oxidizing chemolithotrophs (27). Although phylogenetically diverse, there are common themes. Notably, most of the nitrifiers are closely affiliated with phototrophs: *Rhodopseudomonas palustris* with *Nitrobacter* species, *Rhodocyclus purpureus* with *Nitrosomonas* species, and *Ectothiorhodospira* species with *Nitrosococcus oceanus*. This suggests a close evolutionary link between photosynthesis and nitrite/ammonia oxidation. Both reactions are associated with intracytoplasmic membranes, and related nitrifying and photosynthetic species often share common membrane structural arrangement. Although these data are consistent with the ammonia- or nitrite-oxidizing membrane systems being derivative from photosynthetic membrane systems, the photosynthetic phenotype is widely distributed within the proteobacteria, and phylogenetic affiliation alone is only circumstantial evidence. Thus, these observations should primarily serve as an impetus to better establish the character of the postulated link.

The close connection between photosynthesis and nitrite or ammonia oxidation also suggests the possible existence of ammonia-oxidizing photosynthetic bacteria (10). Ammonia could be used by photosynthetic bacteria as an electron donor in a way analogous to sulfide: 1.3 NH_4^+ + CO_2 = (CH_2O) + 0.65 N_2 + H_2O + 1.3 H^+ (ΔG° = 50 kJ). The possible existence of iron-oxidizing photosynthetic bacteria was postulated earlier on the basis of similar considerations, and representative bacteria were recently isolated (51). The suggested evolutionary progression is consistent with the conversion hypothesis (10–12). This hypothesis proposes that respiratory metabolism, both chemoorganotrophic and chemolithotrophic, has originated repeatedly via the independent conversion of an ancestral membrane-bound photosynthetic apparatus into a respiratory membrane. These conversions presumably occurred as oxidized compounds, produced by anoxygenic and oxygenic photosynthesis, became abundant (11). The purple nonsulfur bacteria and some purple sulfur bacteria are viewed as transitional forms, photosynthetic bacteria with additional (limited) capabilities for aerobic respiration and heterotrophic metabolism (12). The phylogenetic diversity of nitrite-oxidizing and ammonia-oxidizing bacteria seems to reflect the relative ease of transition from photosynthesis to ammonia- and nitrite-based chemolithotrophy. Each nitrifier retains the general structural features of the putative ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, are not derived from an ancestral nitrifying phenotype but appear to have arisen independently multiple times, possibly from different photosynthetic ancestors.

ACKNOWLEDGMENTS

This research was supported by a research grant from the Lyonnaise des Eaux, Paris, France, to B.E.R. and D.A.S.

We thank Stan Watson and Freddy Valois of the Woods Hole Oceanographic Institute for providing cultures and cell material. We thank J. Waterbury for microbiological insights.

REFERENCES

1. **Adkins, J. P., M. T. Madigan, L. Mandelco, C. R. Woese, and R. S. Tanner.** 1993. *Arhodomonas aquaeolei* gen. nov. sp. nov., an aerobic, halophilic bacterium isolated from a subterranean brine. Int. J. Syst. Bacteriol. 43:514–520.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919–1925.
3. **Blumer, M., T. Chase, and S. W. Watson.** 1969. Fatty acids in the lipids of marine and terrestrial nitrifying bacteria. J. Bacteriol. 99: 366–370.
4. **Bock, E., and H.-P. Koops.** 1991. The genus *Nitrobacter* and related genera, p. 2302–2309. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.
5. **Bock, E., H.-P. Koops, B. Ahlers, and H. Harms.** 1991. Oxidation of inorganic nitrogen compounds as energy source, p. 414–430. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.
6. **Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert.** 1990. A new facultatively nitrite oxidizing bacterium, *Nitrobacter vulgaris* sp.nov. Arch. Microbiol. 153:105–110.
7. **Bock, E., H. Sundermeyer-Klinger, and E. Stackebrandt.** 1983. New facultative lithotrophic nitrite-oxidizing bacteria. Arch. Microbiol. 136:281–284.
8. **Bock, E., P. A. Wilderer, and A. Freitag.** 1988. Growth of *Nitrobacter* in the absence of dissolved oxygen. Water Res. 22:245–250.
9. **Böttger, E. C.** 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. 65:171–176.
10. **Broda, E.** 1977. Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. 17:491–493.

11. **Broda, E.** 1978. The evolution of the bioenergetic process. Pergamon Press, Oxford.
12. **Broda, E., and G. A. Peschek.** 1979. Did respiration or photosynthesis come first? J. Theor. Biol. 81:201–212.
13. **Davies, S. L., and R. Wittenbury.** 1970. Fine structure of methane and other hydrocarbon utilising bacteria. J. Gen. Microbiol. 61: 227–232.
14. **Devereux, R., S.-H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. LeGall, and W. B. Whitman.** 1990. Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. J. Bacteriol. 172:3609–3619.
15. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.
16. **Harms, H., H.-P. Koops, and H. Wehrmann.** 1976. An ammonia-oxidizing bacterium, *Nitrosovibrio tenuis* nov.gen.nov.sp. Arch. Microbiol. 108:105–111.
17. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. 139:1147–1153.
18. **Jones, R. D., and R. Y. Morita.** 1983. Methane oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europeae*. Appl. Env. Microbiol. 45:401–410.
19. **Jones, R. D., R. Y. Morita, H.-P. Koops, and S. W. Watson.** 1988. A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. Can. J. Microbiol. 34:1122–1128.
20. **Kalthoff, H., S. Fehr, H. Sundermeyer, L. Renwrantz, and E. Bock.** 1979. A comparison by means of antisera and lectins of surface structures of *Nitrobacter winogradskyi* and *N. agilis*. Curr. Microbiol. 125:375–380.
21. **Klemme, J.-H., I. Chyla, and M. Preuss.** 1980. Dissimilatory nitrate reduction by strains of the facultative phototrophic bacterium *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. 9:137–140.
22. **Koops, H.-P., B. Bottcher, U. C. Möller, A. Pommerening-Roser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. 154:244–248.
23. **Koops, H.-P., B. Bottcher, U. C. Möller, A. Pommerening-Roser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp.nov., *Nitrosomonas ureae* sp.nov., *Nitrosomonas aestuarii* sp.nov., *Nitrosomonas marina* sp.nov., *Nitrosomonas nitrosa* sp.nov., *Nitrosomonas eutropha* sp.nov., *Nitrosomonas oligotropha* sp.nov., and *Nitrosomonas halophila* sp.nov. J. Gen. Microbiol. 137:1689–1699.
24. **Koops, H.-P., and H. Harms.** 1985. Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch. Microbiol. 141:214–218.
25. **Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov.sp. Arch. Microbiol. 107:277–282.
26. **Koops, H.-P., and U. C. Möller.** 1991. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2637. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.
27. **Lane, D. J., A. P. Harrison, D. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace.** 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. J. Bacteriol. 174:269–278.
28. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA 82:6955–6959.
29. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. 21(Suppl.):3021–3023.
30. **Lütters, S., and H. H. Hanert.** 1989. The ultrastructure of chemolithoautotrophic *Gallionella ferruginea* and *Thiobacillus ferrooxidans* as revealed by chemical fixation and freeze-etching. Arch. Microbiol. 151:245–251.
30a. **Mayer, H., J. H. Krauss, T. Urbanik-Sypniewska, V. Puvanesarajah, G. Stacey, and G. Auling.** 1989. Lipid A with 2,3-diamino-2,3-dideoxyglucose in lipopolysaccharides from slow-growing members of *Rhizobiaceae* and from "*Pseudomonas carboxydovorans*." Arch. Microbiol. 151:111–116.
31. **Navarro, E., P. Simonet, P. Normand, and R. Bardin.** 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Arch. Microbiol. 157:107–115.
32. **O'Connor, S. P., M. Dorsch, A. G. Steigerwalt, D. J. Brenner, and E. Stackebrandt.** 1991. 16S rRNA sequences of *Bartonella bacilliformis* and cat scratch disease bacillus reveal phylogenetic relationships with the alpha-2 subgroup of the class *Proteobacteria*. J. Clin. Microbiol. 29:2144–2150.
33. **O'Neill, J. G., and J. F. Wilkinson.** 1977. Oxidation of ammonia by methane-oxidizing bacteria and the effects of ammonia on methane oxidation. J. Gen. Microbiol. 100:407–412.
34. **Orso, S., M. Gouy, E. Navarro, and P. Normand.** 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. 44: 83–86.
35. **Pfennig, N., and H. G. Trüper.** 1989. Anoxygenic phototrophic bacteria, p. 1635–1709. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Schleifer, K. H., and E. Stackebrandt.** 1983. Molecular systematics of prokaryotes. Annu. Rev. Microbiol. 37:143–187.
38. **Seewaldt, E., K. H. Schleifer, E. Bock, and E. Stackebrandt.** 1982. The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. Arch. Microbiol. 131:287–290.
39. **Stackebrandt, E., V. J. Fowler, W. Schubert, and J. F. Imhoff.** 1984. Towards a phylogeny of phototrophic sulfur bacteria—the genus *Ectothiorhodospira*. Arch. Microbiol. 137:366–370.
40. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridisation probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54:1079–1084.
41. **Staley, J. T., and J. A. Fuerst.** 1989. Budding and/or appendaged bacteria, p. 1890–1993. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
42. **Sundermeyer-Klinger, H., W. Meyer, B. Warninghoff, and E. Bock.** 1984. Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrite reductase system. Arch. Microbiol. 140:153–158.
42a. **Swofford, D. L.** 1991. PAUP: phylogenetic analysis using parsimony version 3.1. Computer program distributed by the Illinois Natural Survey. Champaign, Ill.
43. **Trotsenko, Y. A., N. V. Doronina, and P. Hirsch.** 1989. Genus *Blastobacter* Zavarzin 1961, p. 1963–1968. *In* R. G. E. Murray, D. J. Brenner, M. P. Bryant, J. G. Holt, N. R. Krieg, J. W. Moulder, N. Pfennig, P. H. A. Sneath, J. T. Stanley, and S. Williams (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
44. **Watson, S. W.** 1971. Re-isolation of *Nitrosospira briensis* S. Winogradsky and H. Winogradsky, p. 450–456. *In* R. E. Buchanaan and N. E. Gibbons (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
45. **Watson, S. W.** 1971. Taxonomic considerations of the family *Nitrobacteriaceae* Buchanan. Requests for opinions. Int. J. Syst. Bacteriol. 21:254–270.
46. **Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Nitrifying bacteria, p. 1808–1834. *In* R. G. E. Murray, D. J. Brenner, M. P. Bryant, J. G. Holt, N. R. Krieg, J. W. Moulder, N. Pfennig, P. H. A. Sneath, J. T. Stanley, and S. Williams (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
47. **Watson, S. W., E. Bock, F. W. Valois, J. B. Waterbury, and U. Schlosser.** 1986. *Nitrospira marina*, gen.nov.,sp.nov.: a chemolithotrophic nitrite-oxidizing bacterium. Arch. Microbiol. 144:1–7.
48. **Watson, S. W., L. B. Graham, C. C. Remsen, and F. W. Valois.** 1971. A lobular, ammonia-oxidizing bacterium, *Nitrosolobus multiformis* nov.sp. Arch. Microbiol. 76:183–203.

49. **Watson, S. W., and M. Mandel.** 1971. Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. J. Bacteriol. 107:563–569.
50. **Watson, S. W., and J. B. Waterbury.** 1971. Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis* nov.gen. nov.sp. and *Nitrococcus mobilis* nov.gen.nov.sp. Arch. Microbiol. 77:203–230.
51. **Widdel, F., S. Schnell, S. Heising, A. Ehrenreich, B. Assmus, and B. Schink.** 1993. Ferrous iron oxidation by anoxygenic phototrophic bacteria. Nature (London) 362:834–835.
52. **Willems, A., and M. D. Collins.** 1992. Evidence for a close genealogical relationship between *Afipia* (the causal organism of cat scratch disease), *Bradyrhizobium japonicum* and *Blastobacter denitrificans*. FEMS Microbiol. Lett. 96:241–246.
53. **Winogradsky, S.** 1892. Contributions a la morphologie des organismes de la nitrification. Arch. Sci. Biol. (St. Petersburg) 1:88–137.
54. **Winogradsky, S., and H. Winogradsky.** 1933. Etudes sur la microbiologie du sol. VII. Nouvelles recherches sur les organisms de la nitrification. Ann. Inst. Pasteur 50:350–432.
55. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.
56. **Woese, C. R., E. Stackbrandt, W. G. Weisburg, B. J. Paster, M. T. Madigan, V. J. Fowler, C. M. Hahn, P. Blanz, R. Gupta, K. H. Nealson, and G. E. Fox.** 1984. The phylogeny of purple bacteria: the alpha subdivision. Syst. Appl. Microbiol. 5:315–326.
56a. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of purple bacteria: the gamma subdivision. Syst. Appl. Microbiol. 6:25–33.
57. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of purple bacteria: the beta subdivision. Syst. Appl. Microbiol. 5:327–336.